Immunology 1996 **88** 134–139

Chemical carcinogens and antigens induce immune suppression via Langerhans' cell depletion

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SUMMARY

The ability of the chemical carcinogen dimethylbenz(a)anthracene (DMBA) to deplete Langerhans' cells (LC) from murine skin is crucial to the development of antigen-specific suppression. This depletion is a consequence of the LC recognizing the DMBA as antigenic and migrating to the draining lymph nodes to attempt to elicit T-cell activation. This depletion also occurred following exposure to high doses of the contact sensitizers 2,4-dinitrofluorobenzene (DNFB), 2,4,6-trinitrochlorobenzene (TNCB) and fluorescein isothiocyanate (FITC). However, LC depletion was not significant at lower doses, even though these doses were sufficient to induce strong contact sensitivity responses. Application of the contact sensitizer, DNFB, through skin depleted of LC (by pretreatment with either the carcinogen DMBA or the antigen TNCB) failed to induce contact sensitivity. This immune non-responsiveness was antigen specific, and could be transferred by spleen cells to naive mice, which were unable to respond to DNFB. Mouse skin treated with doses of TNCB, that did not cause LC depletion but still induced a normal contact hypersensitivity, retained its ability to initiate a normal immune response to DNFB. Together these findings demonstrate that carcinogens share some properties with antigens as they both cause LC depletion and interact with the immune system. Furthermore, it is this LC depletion, rather than carcinogen treatment, that is a critical factor which leaves the skin immunologically compromised and favours the induction of antigen-specific suppression.

INTRODUCTION

The complete carcinogen dimethylbenz(a)anthracene (DMBA) and the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) cause Langerhans' cell (LC) depletion from murine skin, an important event associated with the pathogenesis of skin cancer.[1,2] This ability to cause LC depletion could be a specific carcinogen-related phenomenon, however, evidence exists that the complete carcinogen DMBA can induce contact sensitivity[3] as well as increasing the rate of LC migration.[4,5] Likewise, the tumour promoter, TPA is able to induce contact sensitivity[6] as well as increasing the rate of LC migration.[5] Consequently this increased migration, as a result of antigen recognition, would result in enhanced antigen presentation in the draining lymph node but would leave the skin depleted of LC. Application of antigen through LC-depleted skin, previously treated with carcinogens, fails to induce an immune response due to the active production of a cellular suppressor signal.[7,8] We demonstrate that LC depletion by either antigens or carcinogens leaves the skin immunocompromised such that application of a further antigen through this depleted skin results in active immune suppression.

Received 17 November 1995; revised 28 December 1995; accepted 8 January 1996.

Abbreviations: CHS, contact hypersensitivity; DC, dendritic cells; DMBA, dimethylbenz(a)anthracene; DNFB, 2,4-dinitrofluorobenzene; FITC, fluorescein isothiocyanate; LC, Langerhans' cells; and TNCB, 2,4,6-trinitrochlorobenzene.

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MATERIALS AND METHODS

Animals

Female BALB/c and C57BL mice, aged 6–8 weeks were obtained from the Central Animal House, University of Tasmania. Female C3H/HeN mice, 6 weeks old, were purchased from the Animal Resource Centre, Western Australia. Mice were caged, fed *ad libitum* and used with approval of the Ethics Committee (Animal Experimentation).

Carcinogens, antigens and vehicles

The carcinogens 7,12,dimethylbenz(a)anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the antigens 2,4-dinitrofluorobenzene (DNFB) and fluorescein isothiocyanate (FITC) were from Sigma. The antigen 2,4,6-trinitrochlorobenzene (TNCB) was from Tokyo-Kasei (Tokyo, Japan). The vehicle for TPA was acetone, for DMBA, TNCB and DNFB 4:1 acetone:olive oil and for FITC 1:1 acetone:dibutylphthalate (A/DBP).

Contact hypersensitivity (CHS) response

The dorsal trunks of mice were shaved and the skin painted with 100 μl of carcinogen/antigen solutions or the relevant vehicles (control). The right ears of both treated and control mice were ear challenged 5 days later with 10 μl of the corresponding carcinogen/antigen. The thickness of the right ear (challenged) and the left ear (unchallenged) was measured 48 hr after the ear challenge using an engineer's spring-loaded micrometer. The percentage increase in ear thickness was calculated according to the following formula:

$$\frac{\text{thickness challenged ear} - \text{thickness unchallenged ear}}{\text{thickness unchallenged ear}} \times 100$$

Secondary skin sensitization was undertaken with the same dose of DMBA 14 days following the primary application and the CHS response was assessed 5 days later.

Detection of transported ^{3}H-labelled DMBA by dendritic cells

The ear skin of BALB/c or C3H/HeN mice was painted with 20 μl G-^{3}H-labelled 7,12-dimethylbenz(a)anthracene toluene solution (^{3}H-labelled DMBA, Amersham International, Amersham, UK; 5 mCi/ml) containing 3 nmol of ^{3}H-labelled DMBA and 100 μCi of radon. Mice were killed 18 hr later and the auricular (draining) lymph nodes, inguinal (non-draining) lymph nodes and the spleen were removed, washed with phosphate-buffered saline (PBS) and mononuclear cell suspensions were prepared by pressing the tissues through a fine mesh sieve and washing in tissue culture medium.

Dendritic cell (DC) enriched populations were separated from the auricular node cells by metrizamide gradient centrifugation[9] by gently layering onto 2 ml metrizamide solution in RPMI-1640 medium containing 5% fetal calf serum (Nygaard, Oslo, Norway; 14·5 g added to 100 ml medium) and centrifuging at 600 *g* for 10 min. Cells that accumulated at the interface (DC) and the pelleted cells (lymphocytes) were collected and washed three times in PBS.

Identical numbers of the collected cell populations were transferred onto filter discs and dried in a 50° oven for 2 hr. The filter discs were then soaked in the scintillant OptiPhase 'HiSafe'II (Wallac, Turku, Finland) and the radioactivity of the cells was counted using a liquid scintillation counter.

Langerhans' cell identification and enumeration

Mice were killed 7 days after cutaneous treatment with antigens or carcinogens. Epidermal sheets were prepared from the treated dorsal trunks using a modified EDTA separation procedure[10] first described by Baker & Habowsky.[11]

The prepared sheets were fixed in acetone for 2 min and then incubated in a culture supernatant containing anti-Ia monoclonal antibody (TIB120, American Type Culture Collection) overnight at 4°. The epidermal sheets were then washed in PBS and incubated with 1:80 diluted horseradish peroxidase-labelled affinity-purified goat anti-rat immunoglobulin G (IgG–HRP, Silenus, Hawthorn, Victoria, Australia) at room temperature for 1 hr. Sheets were washed again in PBS and then the colour of Ia-positive cells was developed with 0·5% 3,3-diaminobenzidene (DAB; Sigma) containing 0·02% hydrogen peroxide, for 10 min. Epidermal sheets were washed three times in cold tap water, lightly dried, and mounted on slides using glycerin jelly.

Ia-positive cells, i.e. LC in the epidermal sheets, were enumerated under light microscopy by counting 12 independent fields and LC number/mm^2 was calculated. All preparations were coded prior to counting.

Induction of immune tolerance by two consecutive antigen applications

Dorsal trunk skin of mice was treated with 100 μl of different concentrations of TNCB (the first antigen) or the vehicle acetone and then, 7 days later, 25 μl of 0·5% DNFB (the second antigen) was applied through the same site. Mice were ear challenged with DNFB 5 days later and the CHS response was assessed a further 48 hr after challenge.

Adoptive transfer of spleen cells

Mice that received the above two consecutive antigen applications were killed 7 days after application of the second antigen (DNFB) and the spleens removed. A single cell suspension was prepared and the number of viable lymphocytes was adjusted to 2×10^7/ml. This cell suspension was intravenously injected into the tail vein of naive syngeneic mice (0·25 ml/mouse). The host mice were then sensitized within 2 hr and ear-challenged 5 days later with DNFB and the CHS response was assessed a further 48 hr later. In the dead cell control group, the spleen cells were snap frozen at −70° and then thawed at room temperature.

Statistical analysis

Differences between control and treated groups were evaluated by Student's *t*-test.

RESULTS

Immune responses induced by carcinogens

A single application of the antigens FITC, TNCB and DNFB and the tumour promoter TPA induced a significant CHS response in BALB/c (H-2^d) mice whereas single application of the complete carcinogen DMBA (1% or 0·1%) failed to induce a CHS response (Fig. 1). DMBA also failed to induce a CHS

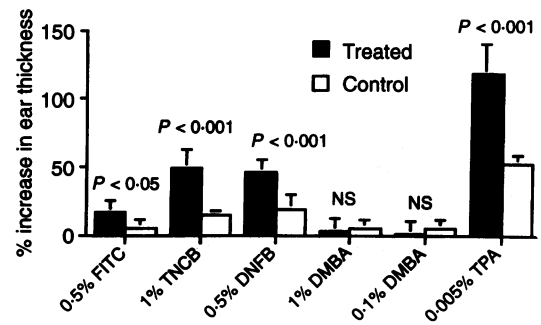

Figure 1. Contact hypersensitivity of BALB/c mice to antigens or carcinogens. Dorsal trunk skin was treated with either the antigen FITC, TNCB, or DNFB or the carcinogen DMBA, or TPA. In control groups, the skin was painted with the relevant vehicle alone and ear-challenged with the corresponding chemicals. Results represent mean ± standard deviation of the percentage increase in ear thickness from six mice per group. Results were compared between the treated and control groups using Student's two-tailed, unpaired *t*-test; NS; no significant difference.

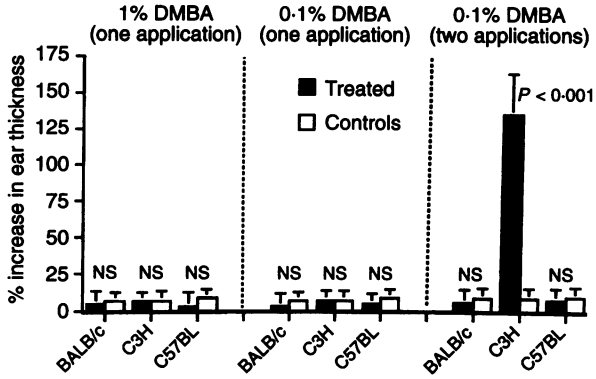

Figure 2. Contact hypersensitivity of mice following one or two applications of DMBA. Dorsal trunk skin of BALB/c, C3H/HeN and C57BL mice was treated with one application of 0·1% or 1% DMBA or two applications of 0·1% DMBA, 14 days apart. The mice were ear-challenged with 0·1% DMBA. In control groups, the skin was painted with acetone, and the mice were ear-challenged with 0·1% DMBA. Results represent mean ± standard deviation of the percentage increase in ear thickness from six mice per group. Results were compared between the treated and control using Student's two-tailed, unpaired *t*-test; NS; no significant difference.

response in C57BL (H-2^b) and C3H/HeN (H-2^k) mice. However, two applications of 0·1% DMBA (14 days apart) induced a strong CHS response in C3H/HeN but not in C57BL and BALB/c mice (Fig. 2).

DMBA transport by dendritic cells

To determine if DMBA was transported from the skin to the draining lymph nodes 20 μl ^{3}H-labelled DMBA (5 mCi/ml) containing 3 nmol of DMBA was applied to mouse ear skin and the cells from the auricular and inguinal lymph nodes and spleen were analysed for the presence of ^{3}H-labelled DMBA. A significantly higher level of radioactivity was detected in the cells of the auricular lymph nodes draining the treated area 18 hr after treatment, than in the cells of the inguinal lymph nodes which drained an untreated area of skin. These results were found with both C3H/HeN and BALB/c (Fig. 3) mice. Radioactivity in the lymphoid cell fraction of the spleen was not detected above background levels.

The nature of the ^{3}H-labelled DMBA-bearing cells in the lymph nodes was analysed following enrichment by metrizamide gradient centrifugation. Analysis of radioactivity showed that the cells in the DC-enriched fraction had a significantly higher count than those in the DC-depleted fraction (Fig. 4). Thus it was the DC that transported ^{3}H-labelled DMBA to the draining lymph node.

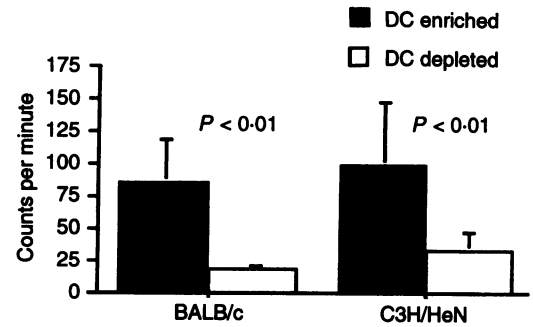

Figure 4. Radioactivity of DC-enriched and DC-depleted auricular lymph node cell preparations (prepared by metrizamide gradient separation) 18 hr after ^{3}H-labelled DMBA ear skin application in C3H/HeN and BALB/c mice. Each sample contained 3×10^5 cells and results represent mean ± standard deviation from six mice per group. *P* values show the comparison between the low-density cells and the pelleted cells (Student unpaired *t*-test). Results were compared between the treated and control groups using Student's two-tailed, unpaired *t*-test.

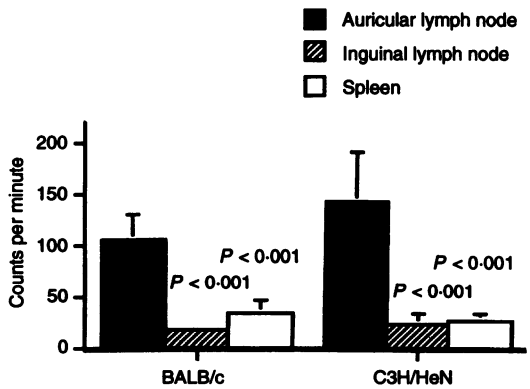

Figure 3. Radioactivity of lymph node and spleen mononuclear cells after ^{3}H-labelled DMBA ear skin application in BALB/c or C3H/HeN mice. Dorsal ear skin was treated with ^{3}H-labelled DMBA, 18 hr later the auricular and inguinal lymph nodes and spleen were removed and single cell suspensions were prepared. Each sample contained 3×10^6 cells and results represent mean ± standard deviation from six mice per group. Results were compared between the auricular (draining) lymph node cells and the inguinal (non-draining) lymph node cells or, between the auricular (draining) lymph node cells and the spleen mononuclear cells (background control), using Student's two-tailed unpaired *t*-test.

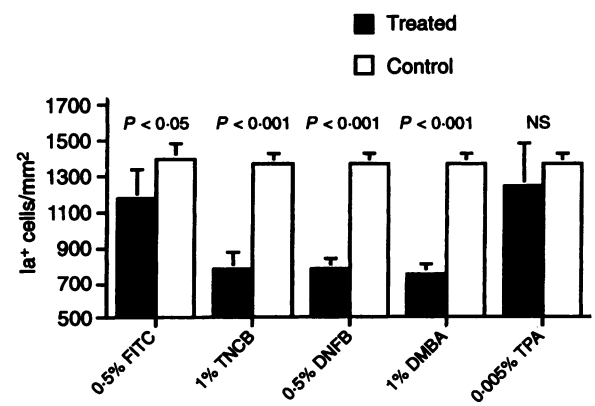

Figure 5. Epidermal LC density following single applications of antigens or carcinogens. A single dose of FITC, TNCB, DNFB, DMBA, or TPA was applied to the dorsal trunk skin of BALB/c mice and 7 days later the epidermal sheets were prepared and stained using the anti-Ia antibody TIB120. Control mice were treated with the relevant vehicle. Results represent mean ± standard deviation of LC densities in the sheets from six mice per group. Results were compared between the treated and control groups using Student's two-tailed, unpaired *t*-test; NS; no significant difference.

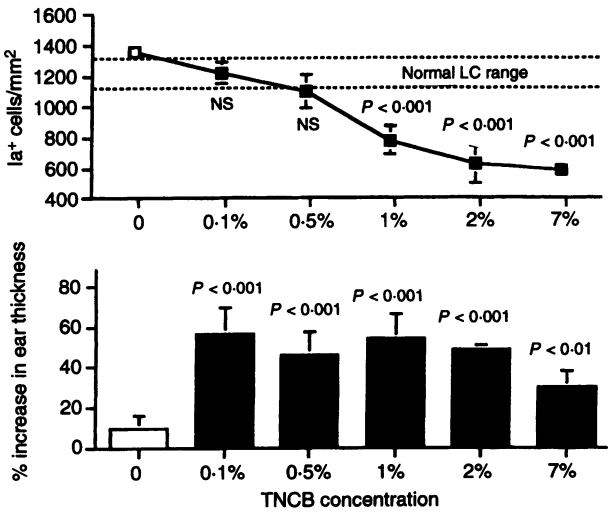

Figure 6. Dose–response curve of LC depletion caused by TNCB in BALB/c mice. Mouse dorsal trunk skin was treated with 100 μl of different concentrations of TNCB. LC in the treated epidermis were enumerated 7 days later (upper panel). Contact hypersensitivity responses were analysed on a parallel series of mice 48 hr after ear challenge with 10 μl of 0·1% TNCB (lower panel). Dose '0' represents control groups where mice were treated with the vehicle acetone. Results represent mean ± standard deviation from six mice per group.

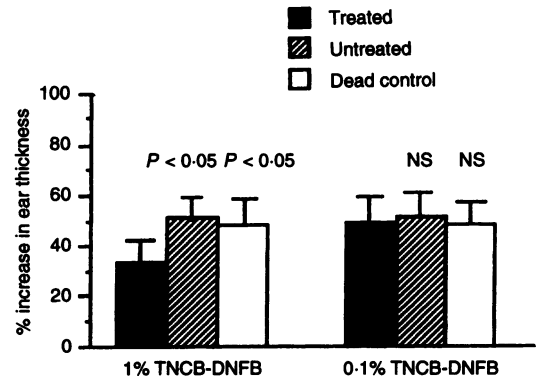

Figure 8. Contact hypersensitivity response to DNFB in mice that received spleen cells from syngeneic donors treated with TNCB, followed by DNFB. The dorsal trunk skin of BALB/c mice was treated with 100 μl of either 0·1% or 1% TNCB followed 7 days later by an application of 25 μl of 0·5% DNFB through the same site. The spleens were then removed and the spleen cell suspension was transferred intravenously into naive syngeneic mice. The hosts were sensitized by 0·5% DNFB 2 hr after the injection and the CHS response assessed a further 5 days later. In the untreated group donor mice were skin-painted with acetone followed by DNFB sensitization. In the dead cell control group the spleen cells were snap frozen at −70° and then thawed at room temperature. Results represent mean ± standard deviation from six mice per group and treated and the control mice were compared using Student's two-tailed, unpaired *t*-test; NS; no significant difference.

LC depletion by carcinogens and antigens

All chemicals except TPA caused a significant decrease in LC number following a single application (Fig. 5). Three weekly applications of TPA were required to cause a significant reduction in LC number (data not shown).

To determine whether a CHS requires a significant depletion of LC from the epidermis, TNCB dose–response experiments were performed. As can be seen from Fig. 6 all the TNCB doses tested (0·1%–7%) caused a significant contact sensitivity response whereas only the higher doses (1%, 2% and 7%) caused a statistically significant reduction in LC number ($P < 0·001$) from the epidermis. The lower doses (0·1% and 0·5%) were still able to cause a contact sensitivity response without causing a statistically significantly depletion in LC from the epidermis. Therefore the induction of a CHS response does not require a statistically significant depletion of LC from the skin.

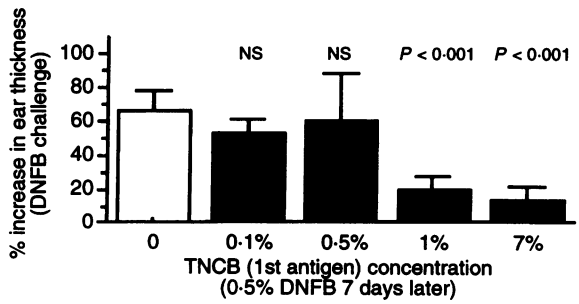

Figure 7. Contact hypersensitivity response to DNFB following application through skin treated with different doses of TNCB. The dorsal trunk skin of BALB/c mice was treated with 100 μl of different TNCB concentrations (first antigen) followed 7 days later by application of 25 μl of 0·5% DNFB (second antigen). The mice were then ear-challenged 5 days later with 10 μl of 0·2% DNFB and the percentage increase in ear thickness was measured. The TNCB dose '0' represents the control groups where mice were treated with the vehicle acetone alone followed 7 days later by DNFB and ear-challenged a further 5 days later with DNFB. Results represent mean ± standard deviation from six mice per group. Results were compared between the treated and control (dose 0) mice using Student's two-tailed, unpaired *t*-test; NS; no significant difference.

Immune tolerance results from LC depletion

Mice treated with LC-depleting doses of TNCB (1% or 7%) failed to elicit a CHS response when DNFB was applied through this TNCB-treated skin (Fig. 7). In contrast, when mice were treated with non-depleting doses of TNCB (0·1% or 0·5%) a CHS response to DNFB was observed when DNFB was applied through this TNCB-treated skin (Fig. 7). The correlation of LC depletion with a failure to induce CHS indicates that tolerance is dependent on a low density of LC.

Suppressor cell generation results from LC depletion

Naive mice that received spleen cells from mice treated with 100 μl of 1% TNCB (which caused LC depletion) followed 7 days later by 25 μl DNFB through the same site, were unable to develop a CHS response to DNFB. In contrast the mice that received spleen cells from the donors treated with 0·1% TNCB (non-LC-depleting dose) followed by DNFB through the same site developed a normal CHS response when sensitized and challenged with DNFB (Fig. 8). Consequently, treatment with a depleting dose of TNCB predisposed the mice for the development of transferable suppression.

DISCUSSION

The complete carcinogen DMBA induces cancer and causes a depletion of the major antigen-presenting cell in the skin, the epidermal LC,[2] owing to a substantial increase in migration of the LC to the draining lymph nodes.[4] The ability of DMBA to increase the migration of LC has two consequences. First, and relevant to the immune response, LC migration provides an opportunity for an immune response to the relevant antigen to be induced. Second, and relevant to the carcinogenic process, significant LC migration leaves the skin depleted of LC and any new antigen arriving at the skin will avoid immune detection as the modified residual LC are incapable of inducing an immune response.[12]

Our observation that DMBA induced a strong CHS in C3H/HeN mice but did not cause CHS in either BALB/c or C57BL mice (Fig. 1 and 2) supports other reports showing that DMBA is a weak contact sensitizer in BALB/c mice,[6] but much stronger in C3H/HeN mice.[3] The failure of BALB/c mice to develop a strong immune response to DMBA could be owing to the failure of either LC to trap the DMBA or to an inability to cause T-cell activation. As shown in Fig. 3, radioactive DMBA was detected in the draining lymph nodes indicating that LC migration was not impaired. Further analysis with metrizamide gradient separation showed that the radioactivity was primarily restricted to the dendritic cell fraction (Fig. 4), indicating that cutaneously applied DMBA was transported by the LC to the draining lymph nodes. This is an important component of the immune response and was detected in both the 'responder' and 'non-responder' mice. Therefore the weak CHS exhibited by the BALB/c mice is unlikely to be owing to the LC but more likely to be associated with the genetics of the response as the BALB/c mice may not produce the appropriate T-cell repertoire to react with major histocompatibility type II-associated DMBA.

The demonstration that DMBA and tumour promoters cause depletion of LC from the epidermis[1,2] to provide an appropriate microenvironment for the induction of tolerance[8,13,14] suggests that this could be a phenomenon specific for chemical carcinogens. This is an important observation because it provides evidence that depletion of LC from the skin is a crucial part of the tumour promotion process. As a result the LC-depleted epidermis will be unable to elicit a protective immune response to any aberrant cells. We have shown here that this phenomenon is not unique to carcinogens as the antigens TNCB, DNFB and FITC, which caused CHS, also depleted LC from the skin (Fig. 1 and 5).

A critical question from the observations that the antigens TNCB, DNFB and FITC, the carcinogen DMBA and, to a lesser extent the tumour promoter TPA, all cause LC depletion (Fig. 5) is why this LC depletion occurs. Cannulation of regional afferent lymphatic vessels in sheep has provided important information on the dynamics of LC migration following exposure to antigens or carcinogens as LC migration is significantly enhanced.[4,5] A further critical observation is that the agents (carcinogens and antigens) which cause enhanced LC migration and LC depletion also induce a CHS response. This increased migration away from the skin as part of the normal immune response to antigen has the potential to leave the skin depleted of LC.

Antigen dose–response experiments revealed that LC depletion is not an essential requirement for inducing an immune response as low doses of TNCB induced a strong CHS response in the absence of statistically significant LC depletion (Fig. 6). However, in response to antigen an elevated rate of LC migration from the skin through the draining lymphatics has been observed.[15] This increased rate of LC migration is sufficient to induce an immune response but does not necessarily lead to depletion of LC from the epidermis. The consequence of this is twofold. First, as LC are powerful antigen-presenting cells, only a small number would be required to initiate a successful immune response; and second, sufficient LC remain in the epidermis to facilitate an effective immune response to a subsequent antigen. This would be beneficial to the host as an immune response is generated without depleting the skin of LC and therefore the potential for immunosuppression is minimized. In contrast, high doses of antigen or carcinogen result in a massive increase in LC migration which leaves the epidermis depleted of LC and therefore immunologically compromised.

The density of LC is crucial for the induction of an immune response and application of antigen through skin naturally deficient in LC, such as tail skin in mice,[16] or application of antigen through skin depleted of LC, due to prior treatment with DMBA,[8,17,18] ultraviolet light,[16,19,20] or TPA[13,14] fails to induce an immune response. The requirement for a critical LC density is well demonstrated following exposure to the antigen TNCB as LC-depleting doses of TNCB prevented a CHS response to a second antigen, DNFB, when this second antigen was applied through the same site (Fig. 7). This did not occur if the initial dose of TNCB was insufficient to cause LC depletion but could still induce a CHS response.

Application of high concentrations of antigen provides evidence to support the proposal that it is the LC-depleting ability, rather than the carcinogenic activity, that accounts for the generation of tolerance. In addition the presence of a cell-mediated suppressor signal was identified in the spleen, as adoptive transfer of spleen cells prevented the induction of a CHS response (Fig. 8). These findings therefore support the proposal that it is the LC depletion, rather than prior treatment with the carcinogen, that is responsible for tolerance induction and that LC depletion will occur as part of an over-reactive response to high doses of antigens or carcinogens. The ability of antigens to cause LC depletion poses the intriguing possibility that they may also function as tumour promoters.

ACKNOWLEDGMENTS

This work was supported by the Tasmanian Cancer Committee and M. Qu was the recipient of a University of Tasmania Writing Up Scholarship.

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